The Network of Time

Roenneberg, Till; Merrow, Martha

Published in:
Current Biology

DOI:
10.1016/S0960-9822(03)00124-6

Publication date:
2003

Current Biology, Vol. 13, R198–R207, March 4, 2003, ©2003 Elsevier Science Ltd. All rights reserved. PII S0960-9822(03)00124-6

The Network of Time: Understanding the Molecular Circadian System

Review

Till Roenneberg and Martha Merrow

The circadian clock provides a temporal structure that modulates biological functions from the level of gene expression to performance and behaviour. Pioneering work on the fruitfly Drosophila has provided a basis for understanding how the temporal sequence of daily events is controlled in mammals. New insights have come from work on mammals, specifically from studying the daily activity profiles of clock mutant mice; from more detailed recordings of clock gene expression under different experimental conditions and in different tissues; and from the discovery and analysis of a growing number of additional clock genes. These new results are moving the model paradigm away from a simple negative feedback loop to a molecular network. Understanding the coupling and interactions of this network will help us to understand the evolution of the circadian system, advance medical diagnosis and treatment, improve the health of shift workers and frequent travellers, and will generally enable the treatment of clock-related pathologies.

Introduction

Most cultures have proverbs that extol the virtues of rising early, such as “the early bird gets the worm”, “Morgenstund hat Gold im Mund“ or “l’avenir appartient a ceux qui se levent tôt”. But not everyone adheres to this wisdom. Recall this familiar scene: a mother and her younger son cheerfully chat and heartily devour their breakfast, while father sips his coffee silently, and the teenager’s bodily presence is a mere travesty of the physiological state called ‘awake’. This scene illustrates the phenomenon of the ‘chronotype’, a term that refers to the individual scheduling of behaviours to certain times of day.

At the base of these behaviours lies the biological clock or the circadian system, which is found in organisms of all phyla. The term circadian, literally ‘about one day’, refers to the observation that the endogenous day is generally slightly shorter or longer than 24 hours when the biological clock ‘free-runs’ in constant conditions, shielded from all environmental time cues (zeitgebers). In free-run conditions, the temporal sequence of endogenous events proceeds essentially unchanged; those events that are normally scheduled to the light period occur in the ‘subjective day’, and those that normally take place in darkness occur in the ‘subjective night’.

Institute for Medical Psychology, University of Munich, Goethestrasse 31, 80336 Munich, Germany.
E-mail: till.roenneberg@imp.med.uni-muenchen.de

Different centres that control circadian physiology have been localised in the nervous systems of many animals, from cockroaches to mammals. In humans, this centre resides a couple of centimetres behind the bridge of the nose, in a pair of nuclei above the crossing of the optic nerves. Each of these ‘suprachiasmatic nuclei’ (SCN) is only about the size of a grain of rice, but their qualities are remarkable. Individual rat SCN cells in culture exhibit a circadian rhythm in spontaneous firing rate that appears to be sustained indefinitely [1]. Through their coupling, these cellular clocks acquire stunning functional properties, such as the ability to activate or silence genes throughout the body at the appropriate times, or to modulate our senses and behaviour. When SCN tissue is cross-transplanted between two animals, circadian qualities are carried along [2]; for example, the activity–rest cycle of the recipient reflects the period of the donor.

The SCN thus appears to be responsible for organising endogenous daily programmes throughout the body. When it became clear that isolated body parts of insects are able to produce circadian rhythms [3-5], however, researchers looked at cultured mammalian cells, such as rat fibroblasts, and found that they also exhibit circadian gene expression [6]. In tissues as different as brain, heart, muscle or lung [7], a similar set of ‘clock genes’ undergo oscillatory changes in expression level. The SCN ‘pacemaker’ and these organ clocks have different qualities, however, forming a hierarchy within the circadian system. The same genes whose expression levels reach a maximum in the early morning in the SCN do so several hours later in the periphery. While the SCN rhythms continue indefinitely, the organ clocks appear to dampen within a few days [7]. When rats are subjected to a ‘jetlag’ experiment, in which the light:dark cycle is shifted by several hours, rhythms shift with different speeds in different organs. While the SCN apparently adjusts within one cycle, the liver can take more than six days to synchronise with the new light:dark cycle. This was a surprising observation, because the mammalian activity–rest rhythm is an output of the SCN [8] and takes several cycles to adjust to a shifted light regime [9,10].

Large-scale screens using gene arrays showed that numerous genes, beyond the known clock genes, are circadianly regulated in different organs and tissues [11–13]. These so-called clock-controlled genes represent the output pathway of the circadian system. They facilitate the daily modulation of many physiological properties, such as blood pressure (lowest just after midnight), mental performance (best in the mid-afternoon), or hormones (cortisol is highest in the morning, melatonin at night). Recently [14], disruption of a clock gene in the mouse was found to be associated with increased risk of irradiation-induced tumorigenesis, perhaps as a result of loss of normal circadian controls of genes concerned with regulation of cell proliferation and the cell cycle.

Thus, underlying circadian behaviour is a molecular machinery that is present in practically all body cells, and the daily temporal structure of behaviour appears to be the product of a hierarchical amalgam of brain and peripheral clocks. We are just beginning to understand how this organisation is effected. What follows is a synthesis of our current knowledge of the mechanisms that generate this systematic and plastic temporal programme.

The Art of Entrainment

A man who we shall refer to as Mr. McGee has the impression that he is awake for several days in a row, and at other times he sleeps for days. He has been in and out of psychiatric care for more than a decade, mainly for treatment of depression. Analysis of his diary, which documents the exact times of all his daily events for many years, reveals that his sleep–wake cycle is not properly synchronised to the 24 hour day, even though he is sighted. On average, Mr. McGee appears to live a 25 hour day (Figure 1). His sleep–wake cycle shares features with those of free-running individuals who are isolated from all entraining signals, or zeitgebers. Occasionally, he appears to approach a 24 hour rhythm for several days, but then he breaks loose again (see 'partial entrainment' in Figure 1).

This 'relative coordination' [15] is a typical feature of a circadian rhythm that does not receive a strong enough zeitgeber [16], and it turns out that Mr. McGee hardly ever leaves his dimly lit room. Many blind people suffer from similar sleep patterns because their circadian system cannot be synchronised to the light:dark cycle [17]. Although there are many possible reasons for Mr. McGee's irregular sleep patterns, it is likely that the lack of any strong day–night difference in his light exposure contributes to the symptoms. When Mr. McGee was in the hospital (first three weeks in Figure 1), he experienced a stronger zeitgeber and managed to keep more or less to a regular 24 hour sleep pattern, although he never fell asleep before 3 a.m.

The synchronization of a circadian system is an active process called 'entrainment', with light being the predominant zeitgeber [18]. Although circadian systems are generally investigated in constant conditions, the function of the biological clock in nature is entirely reflected in entrainment. It is the regular alternation between night and day that has shaped the evolution of the circadian clock. Thus, the clock's ability to oscillate without a zeitgeber is a reflection of how the system has evolved to work optimally when it is synchronised to the environment.

In mammals, unlike in other animals, light reaches the circadian system exclusively through the eyes [19]. Mice that lack all rods and cones can still be entrained by light [20]. The race to identify the responsible light receptors — which also influence a number of other processes, such as pupillary constriction, melatonin suppression and adaptation of the primary visual system [21–23] — has reached the final laps [24–26]. The most recent contributions [27,28] have shown that melanopsin plays a role in circadian light entrainment, but acts with other photopigments that are still to be discovered.

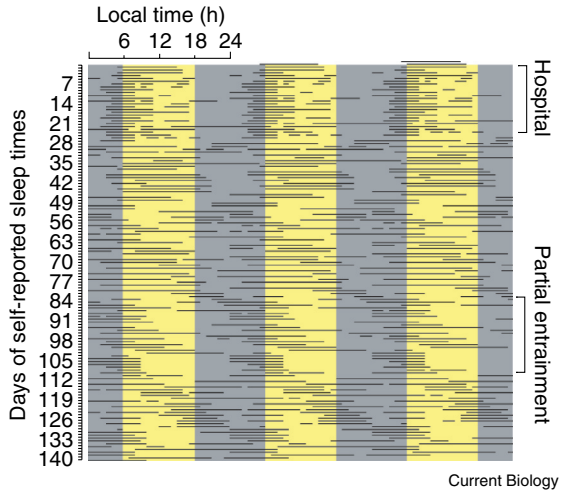

Figure 1. Self-assessed sleep times of a man with strange sleeping habits based on his diary.

The black bars represent the times when the man slept. The original data are plotted three times, so that the rhythm can be followed across midnight. Each line represents three days; for example, the first line shows the sleep times for days 1, 2 and 3; the second line for days 2, 3 and 4; and so on. A theoretical light period from 6 a.m. to 6 p.m. is drawn to indicate local time. (Based on our unpublished data.)

Given the systematic way that endogenous, free-running rhythms are entrained by light:dark cycles, pioneering circadian researchers compared circadian clocks to physical oscillators [29]. When one oscillator entrains another — for example, when a biological clock is entrained by the sun — their relative phase relationship depends on their respective endogenous periods. This means the shorter the free-running period of the circadian clock, the earlier is its phase relative to the entraining day. Individuals may have different free-running periods, for example because of genetic differences (Figure 2A), and it has been shown that those who like to go to sleep and get up early tend to have a shorter free-running period than those who prefer to sleep later [30] (Figure 2B).

The free-running period is not, however, the only factor that determines the phase of entrainment; another determinant is the strength of the zeitgeber, for example the amplitude of day–night light intensity differences (compare Figure 2B and 2C). The effect of decreasing zeitgeber strength on the phase of entrainment again depends on the individual's free-running period. If the free-running period is shorter than 24 hours, the clock will move forward to an earlier time with decreasing zeitgeber strength. With an endogenous period longer than 24 hours, typical for most humans, the clock will move sleep and activity to a later phase [31]. Thus, extreme chronotypes at both (early and late) ends of the spectrum will become even more extreme when the strength of the zeitgeber is decreased.

In some humans, the phase of entrainment is so extreme that it leads to syndromes known as advanced

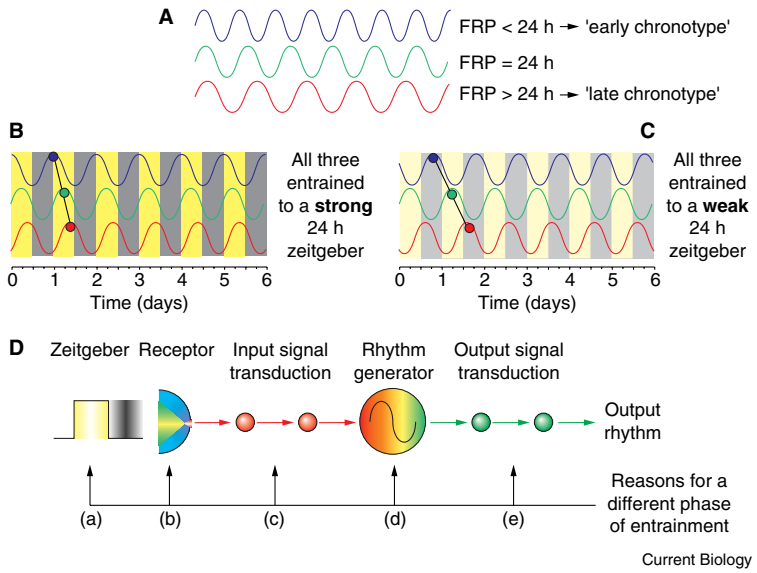

Figure 2. The biological clock is synchronized to the 24 hour day by an active process called entrainment.

The circadian clocks of different individuals can have different periods when deprived of all exogenous time cues (zeitgebers). The so-called free-running periods (FRPs) can be slightly shorter or longer than 24 hours (A). Simple oscillations are drawn to exemplify clocks with different free-running periods. Because of the mechanisms of entrainment, clocks with different free-running periods will synchronize with different relationships to a 24 hour light:dark cycle. These differences in 'phase of entrainment' depend both on the free-running period and on the strength of the zeitgeber (B and C). The circadian system is often depicted as a pathway from the input that receives the zeitgeber signals to the output that controls the observable rhythms, with the mechanism that generates the circadian rhythmicity at its centre (D). The phase of entrainment may vary because of differences occurring at any stage of this pathway (a–e).

or delayed sleep phase syndrome (ASPS and DSPS). These patients, respectively, regularly wake up as early as 4 a.m. or cannot fall asleep until 3 a.m. [32–34]. Although the tendency for a specific chronotype has a genetic basis, it can also change during development. In general, teenagers shift to chronotypes that are later than in childhood or adulthood [35,36].

The 'phase of entrainment' can thus vary for different reasons. If the circadian system is depicted as a pathway with the mechanism that generates the circadian rhythmicity at its centre (Figure 2D), the rhythm generator receives the entraining signals via a specific receptor and transduction pathway and controls output rhythms by sending signals down an-other transduction pathway. Two individuals can be different chronotypes simply by exposing themselves to very different zeitgeber strengths ('a' in Figure 2D). Alternatively, they might respond differently to identical light signals, for example because of genetic differences in their light receptor (b) or transduction cascade (c). Their clocks may have different free-running periods (d), again as a result of genetic variation, while the available light regime and the phototransduction pathways are identical. Finally, the way their circadian clocks control their output might be different (e).

The pathway shown in Figure 2D can be applied equally to the whole organism or to a single cell. In the former case, the receptor resides in the retina and the rhythm generator in the SCN. In the latter case, the receptor is inherent to the cell, and the rhythm generator consists of molecular feedback loops. For the whole organism, the zeitgeber is exogenous (light, for example), while the entraining signals for cellular clocks are endogenous factors, such as transmitters or hormones. In the case of the liver clock, both signals from the SCN and cues from feeding and metabolism contribute to entrainment [37,38]. The nature of entrainment is thus distinct for different tissues, which may be adaptive, helping the individual to adjust to different timing of food sources or to changing photoperiod and seasons, or, in modern times, to new time zones or work schedules.

The Rise and Fall of Simple Negative Feedback

The similarity between a simple mechanical oscillator and the biological clock suggested that the mechanism behind circadian rhythmicity would also be simple. From research on unicellular organisms, it has long been known that single cells are capable of generating circadian rhythms [39], but proof that multicellular organisms have a cell-based circadian mechanism only came in the 1990s, first for a marine mollusc [40] and then for single neurons from the mammalian SCN [1].

The first 'clock' gene to be discovered, the *Drosophila Period* (*Per*) gene, was identified in a mutant screen using circadian read-outs [41]. Analysis of the kinetics of this gene's expression led to a simple model [42] that still works for all genetic model systems used to study circadian rhythms (Figure 3A). A gene is transcribed and translated into a protein. The protein directly or indirectly inhibits its own transcription, and the cycle restarts when the protein is degraded. The result is circa-24 hour oscillations of RNA and protein, as observed for many clock genes and their products. This molecular loop is a simple negative feedback with several components, each of which depends on the previous component in the loop; for example, the rate of protein production depends on the RNA level.

The initial feedback model for *Drosophila* involved just one gene and its two components, RNA and protein (with question marks, which the authors had the foresight to include in their scheme) [42]. Although many more components have since been discovered, they apparently are all parts of the same simple transcription–translation feedback loop, functioning as activators, inhibitors or kinases. In mammals, the first breakthroughs came with mutants, the first being a mutant hamster found by chance to have a short

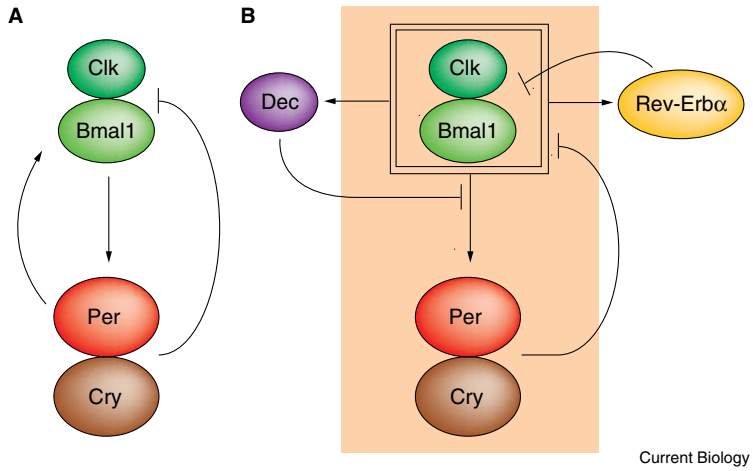

Figure 3. Models of the molecular circadian machinery.

Until recently, the model described a simple negative feedback with several components. (A) In mammals, these involve the *Per* and *Cry* genes and their activators Clk and Bmal1. In addition to the negative feedback, there is a positive feedback of Per2 on *Bmal1* expression. (B) Recent results, including discoveries of additional components indicate that the 'simple' feedback with a negative and a positive arm is much more complex than first thought. In the different interactions between the circadian clock genes, the dimer Clk–Bmal1 appears to be the common activator (rectangle in B). For reasons of simplicity, only the proteins are drawn in the diagrams. Note that the inhibitory effect of Per–Cry on Clk–Bmal1 activation affects all three loops. (For details, see text.)

period [43]. Years later, a mouse carrying a mutation in the *Clock* (*Clk*) gene was recovered in a large-scale mutant screen [44,45]; the subsequently cloned gene was predicted to encode a transcription factor [46].

Mammalian homologs of the *Drosophila Per* gene were later identified using available genome sequences [47] and a clever homolog search [48]. The discovery that very different animals share clock genes jump-started the mammalian field. Until recently, the 'core' of the mammalian molecular loop comprised three *Per* genes, two *Cryptochromes* (*Cry*), *Clk* and a gene called *Bmal1* [49]. How these genes and their products work together has been elucidated by a combination of cell transfection experiments and genetics. The cell-culture approach used reporter genes under the control of a clock-regulated promoter, or the yeast two-hybrid assay of protein–protein interactions (see [50–52], for example). The genetic approach used clock mutants to investigate the expression patterns of the other known clock components (see [53,54], for example).

The results showed that *Per* and *Cry* expression is activated by Clk–Bmal1 dimers, which bind to a specific promoter sequence — the 'E-box', CACGTG — while Per and Cry proteins dimerise and inhibit their own transcription by influencing Clk–Bmal1 activation [46,55]. Cry provides the repressor function [50], possibly by modifying histone acetylation [56], while the role of Per in the context of negative feedback is not yet entirely clear — it might be involved in nuclear and/or cytoplasmic translocation [57] of the Cry–Per dimers or stability of the Cry protein. Besides the negative feedback, a positive effect was found for the Per2 protein on Bmal1 levels [58].

From these results, the mammalian molecular clock was initially modelled as an autoregulatory, negative feedback transcription–translation loop with interlocking positive and negative arms [49,58–60] (Figure 3A). But subsequent data have led to elaboration of this loop. It is clear, for example, that the three Per and two Cry homologs can mix and match to form different complexes [50]. Furthermore, new components, such as Rev–ErbBα or Dec1 and Dec2 (Figure 3B), have recently been described. Rev–Erbα, an orphan nuclear receptor, has a negative regulatory effect on Bmal1, adding a negative circuit to the central loop(s) [61]. Dec1 and Dec2 are members of the basic-helix–loop–helix transcription factor family [62,63]. Their protein levels vary rhythmically in the SCN and other tissues, and this oscillation is mediated by Clk–Bmal1. Dec1 and Dec2 both interact negatively with Clk–Bmal1-mediated transcriptional activation. The original simplicity of the feedback loop has thus disappeared.

There is no doubt that the clock components and negative feedback loops that have been described in species as different as fungi and mice are essential parts of circadian systems. Although different species might use different proteins, or use orthologous proteins in completely different roles, most circadian systems use a similar formula of interlocking feedback loops. But the increasing complexity of the feedback model warrants reexamination. A simple circular loop (Figure 4A) invokes certain predictions: all components oscillate with the same period; they adjust to a shifted light:dark cycle with the same speed; and with any one of the components eliminated, the system should be arrhythmic under all conditions. Three recent sets of experiments have produced results that challenge the predictions made by a simple loop model.

In the first set of experiments, *Per* and *Cry* gene expression in the SCN was examined in jet-lag experiments [10]. The RNA and protein products of these genes were found to adjust to an advance of the light:dark cycle — as occurs, for example, following an east-bound flight — with different kinetics. In retrospect, this is not surprising. *Per1*, for example, is regulated both by the clock — via Clk–Bmal1 — and the light signalling pathway — via posphorylation of the transcription factor CREB on residue serine 142 [64]. The mechanism of light regulation of *Per2* has so far not been defined, but it is clearly not the same as for *Per1*, and the *Cry* genes are not light-induced and each is apparently regulated differently [50]. In spite of these complications, the *Cry* and *Per* genes are often drawn in model diagrams as though regulated like an operon — that is, controlled by a common promoter.

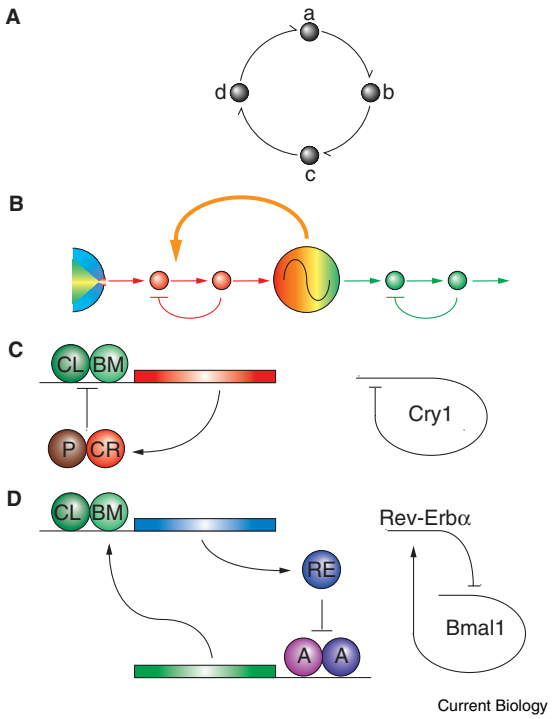

Figure 4. Qualities and taxonomy of molecular feedbacks.
(A) Simple, multi-component feedback loops make certain predictions about the individual components that are challenged by experimental results (see text for details). (B) Circadian pathways include feedbacks outside of the presumed rhythm generator, both in the input and output pathways as well as feedbacks from the rhythm generator back onto the inputs. (C,D) The individual clock genes in the mammalian circadian system form either single-gene-feedback-loops (C) or two-gene-feedback-loops (D). CL, Clock; BM, Bmal1; P, Per1 or Per2; CR, Cry1 or Cry2; RE, Rev-Erbα; A, unknown activator(s).

In the second set of experiments, clock gene mutant mice were examined, not only in constant darkness, but also in constant light. Many of these mutant mice were found to become arrhythmic in constant darkness, but some of them remain rhythmic in constant light [65]. The third set of experiments investigated clock gene double mutants: a combination of *Cry2* and *Per2* mutations was found to rescue rhythmicity in mice, while the *Per2* single mutant is arrhythmic [66].

Results showing the limitations of a simple feedback model are not a special feature of work on mammals. Experiments in *Neurospora* demonstrated that qualities of the circadian clock remain intact in strains with functional or genetic knock-outs of clock genes [67–69]. Furthermore, *Neurospora* clock genes encode components of the light-input pathway, including a blue-light receptor [70–72]. Such a close association of clock and light-input components has also been found in higher plants [73], where mutations in phytochrome and cryptochrome photoreceptors cause altered free-running periods in constant light [74]. Theoretical modelling shows that the observed phenotypes of clock mutants — arrhythmicity, altered period length or loss of temperature compensation — could result from clock components functioning as a circadianly controlled input pathway [75,76] (Figure 4B). And the work on many different model systems suggests that a number of clock components remain to be identified.

Building a Molecular Network

Negative feedbacks are common regulatory mechanisms facilitating adaptation, simple product inhibition or saturation in many biochemical or neuronal pathways [75]. Circadian pathways also contain feedbacks beyond those presumed to constitute the central rhythm generator (Figure 4B). The input pathways which transmit zeitgeber signals to the clock, for example, are themselves under circadian control in most organisms [77–80]. This creates a fuzzy border between rhythm generator and input pathways: while the inputs change the qualities of the clock, the clock changes the properties of the input. So how do we know whether a negative feedback identified as part of the circadian system actually generates the circadian rhythmicity or is a control feedback within the circadian pathway?

Among the many feedbacks involved in the cell's biochemistry, those capable of generating self-sustained rhythmicity are surely an exception. But even if they apparently do have this ability, as in the case of the clock loops that have been discovered, it is difficult to judge whether they accomplish this on their own or as part of a much larger network. The current molecular model leaves many important questions unanswered. How can a transcription–translation feedback generate periods in a 24 hour time frame? How extensive is the network necessary to produce a circadian rhythm? And what was the function of these loops before a circadian system evolved?

Complex networks of feedbacks must have existed even in the earliest cellular organisms. We have recently modelled the possibility that the circadian molecular machinery evolved from a network of many coupled feedback loops [81]. Circadian biologists distinguish between driven and self-sustained rhythms. While the former are exogenously evoked and cease in constant conditions, the latter continue. Modelling complex feedback networks suggests, however, that even non-circadian network systems are not simply driven by regular stimuli. Chaotic responses are prevented in the model by making the input pathway sensitive to both the external environment and the endogenous state of the system, similar to what is found in most circadian systems (see thick orange arrow in Figure 4B). This drivable network still does not behave like a circadian clock, but it can easily be turned into such a mechanism by changing the coupling strength between the network components.

The model also offers an explanation for how a biochemical system can oscillate with a self-sustained periodicity as long as 24 hours. All of the contributing feedback loops, when isolated, have periods shorter than 6 hours and dampen down within a couple of cycles, but when they are appropriately connected to a network they produce a self-sustained, circa-24 hour rhythm. When components similar to those found in circadian molecular feedback loops have been

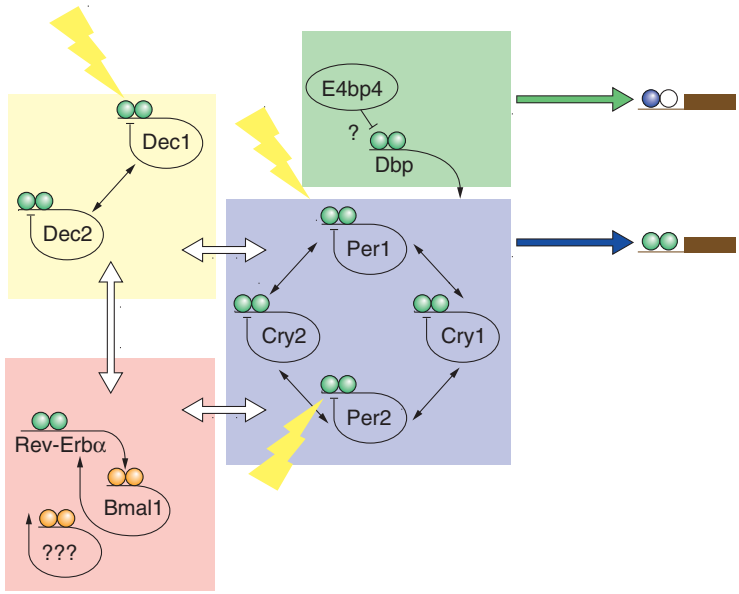

Figure 5. The circadian machinery as a network of interacting feedback loops. Each clock gene is drawn as a single, negative autoregulatory feedback loop. The individual feedbacks are coupled by sharing proteins in the transcription factor complexes. The green spheres indicate the dimer Clk–Bmal1; the yellow spheres represent an as yet unknown enhancing transcription factor. The genes constituting the mammalian orthologues of the first clock genes discovered in *Drosophila*, *Per* and *Cry*, are grouped within the blue box. This feedback network affects several others. The output regulator Dbp apparently feeds back onto the system (green box, see text for details). The two Dec protein feedbacks (yellow box) and the Rev–Erbα loop (red box) are coupled by sharing transcription factors. Further elements and feedbacks within this growing network are to be expected from the identification of the activators of *Bmal1* (yellow spheres) and from insights into the interaction between the rhythmic PAR family transcription factors Dbp and E4bp4. At least Clk–Bmal1 (blue arrow) and Dbp (green arrow) drive the rhythmic expression of output genes. So far, three genes in this network, *Per1*, *Per2* and *Dec1*, have been shown to be light inducible. The network only shows elements of the molecular circadian system which are known to form transcription/translation feedback loops and to oscillate with a circadian period. Other important components, such as kinases [105], or genes with unclear clock function, such as *Per3* [106], are not included.

investigated in a different cellular context, they have been found to cycle with a short rhythm. This was the case, for example, with the transcription factor Hes1 — related to the Dec proteins [62] — which was found to cycle with a 2 hour period [82]. Similar short periods have been observed for other transcription–translation feedback loops [83, 84].

The kinetics and detailed regulatory mechanisms operating at clock gene promoters are indicative of highly specific control, in spite of shared enhancers and inhibitors. The kinetics of *Per1* and *Per2* light induction, for example, are very different [85,86], and the two transcripts and proteins oscillate out of phase in constant darkness [47,87]. The regulation of each clock gene can, therefore, be formally regarded as an individual negative autoregulatory feedback loop. In this scenario, the *Cry*, *Per* and *Dec* genes each form a single-gene feedback loop (Figure 4C). The protein product of each of these genes facilitates the down-regulation of its own transcription. There may be differences in the type of interaction: Crys may directly facilitate inhibition [50], while Pers may have cofactor function. But the net effect, controlled self-inhibition of transcription, is the same.

The *Bmal1* expression cycle can be represented as a two-gene feedback loop (Figure 4D). Bmal1 enhances the transcription of the *REV–Erbα* gene, but Rev–Erbα negatively regulates *Bmal1* expression. Still unknown in this scheme is the activator of *Bmal1* transcription, but as there are several transcriptional activators in the Rev–Erbα family, it may turn out to be one of these [61].

The consequence of treating each clock gene as an individual feedback loop leads to a network of loops which, together, form the molecular machinery of a circadian clock (Figure 5). In this network, all the feedbacks are coupled with each other, either directly or indirectly, for example by sharing proteins in their transcription factor complexes or by contributing to negative regulation of other feedback loops. The coupling strength between two feedback loops may be very specific and may even change under certain conditions, such as constant light versus constant darkness or with different nutrients. The arrangement of the network into different domains (coloured areas in Figure 5) has mainly historical reasons and does not necessarily imply specific functional groups of feedbacks, although functional domains undoubtedly will be defined experimentally.

There are some indications as to which additional domains are likely to be described soon. The factors that regulate known clock components will link the existing network to a host of other genes which are also regulated by the expanded network. The activators of *Bmal1* are candidates for linking the molecular clock network to the family of orphan nuclear receptors [61]. The D-box binding protein (Dbp) is another candidate. Dbp was primarily considered an output of the clock, because it shows robust circadian rhythms [88,89]. However, Dbp mutant mice have a shorter free-running period [90], indicating that it is part of a feedback with the clock. Like Dbp, other members of the PAR family of 'proline and acid amino acid rich' transcription factors, such as E4bp4 [91], are rhythmically expressed. These rhythmic transcription factors could form another domain of feedback loops.

Why has the existence of an extended circadian network remained elusive to date? There are probably

numerous reasons, including the impracticality of performing saturating mutant screens on mammals, the possible involvement of essential genes, and the limitations of laboratory protocols that screen for a discrete set of circadian qualities. Given the characteristics of mouse circadian behaviour, a subset of clock mutants would be difficult to pick up in light:dark cycles, because mice show an acute response to light — they stop moving — that obscures determination of phase angles during the daytime.

Predictions from a Circadian Network

A circadian network helps to explain observations that are incompatible with a single central loop. Thus, elimination of a network component in a mutant may lead to arrhythmicity under a given condition, such as constant darkness, while the network is able to rearrange itself and rescue circadian and self-sustained rhythmicity in another condition, for example constant light [65]. While a single mutation may create imbalances in the network that lead to arrhythmicity, the elimination of an additional feedback in a double mutant may provide a state of the system that rescues circadian rhythmicity [66].

Networks in different tissues of the same organism, or in different species, may share the same genes but the coupling of the feedback loops may be different, and the feedbacks may serve different functions within the system. The Cry feedback, for example, may be integrated into the network as part of the light input — as in the lateral neurons of the *Drosophila* brain [92] — or as a feedback mechanism that ensures self-sustained rhythmicity — as in *Drosophila* peripheral tissues [93] or in mammals [50,94] — merely by being coupled differently to the rest of the network.

It is also conceivable that different parts of the network each adopt their own circadian dynamics under special experimental conditions and start to oscillate independently. Under these conditions, it would be possible to observe two, more or less independent circadian oscillations when recording different outputs of the system. The existence of independent circadian rhythms within single cells has already been shown for the unicellular alga *Gonyaulax polyedra* [95]. Identifiable domains of the network could contain feedbacks that are important for different aspects of the circadian system. These might include those feedbacks that make the oscillation robust, that relay circadian control to the outputs, that are sensitive to the intracellular milieu (for example to the redox potential [96]) or that are mainly sensitive to environmental stimuli, such as light.

In mice, light appears to affect the mammalian circadian network via the induction of three genes: *Per1* [85], *Per2* [86] and *Dec1* [62] (light flashes in Figure 5). Light received via retinal photoreceptors [97] is transmitted to the SCN via two pathways: directly via the retino-hypothalamic tract, using glutamate and pituitary adenylate cyclase activating polypeptide (PACAP) as principle neurotransmitters; and indirectly, via the intergeniculate leaflet and the midbrain, using γ amino butyric acid (GABA), neuropeptide Y, and serotonin as transmitters [49]. All three light-induced clock genes are responsive only at specific circadian times. *Dec1* is light-induced throughout the subjective night [62]; *Per1* both at the beginning and the end of the subjective night [85]; and *Per2* only in the early subjective night [86]. Also, the induction kinetics of the three genes are distinct. The intracellular transduction pathways appear to involve Ca^{2+}-mediated phosphorylation of CREB, which binds to CREs in the promoters of *Per1* and *Per2*. An important open question is how the different input genes fine-tune the light responses of the circadian clock. All three may be responsible for different light-responses, such as phase delays, phase advances, changes in period [65] or the measurement of day length, by separately responding to dawn and dusk [98].

The outputs of the network could be regulated by the interactions between the clock gene activators and inhibitors, which could control any number of genes via appropriate promoter sequences (large arrows in Figure 5). The vasopressin gene is rhythmically activated by Clk–Bmal1 binding to an E-box sequence in its promoter [99]. In other cases, clock-associated regulatory sequences are still to be defined. Microarray data have shown that up to 10% of gene expression in a given tissue is under circadian control [11–13]. Given that a different set of output genes is circadianly regulated in each tissue it is possible that, across all tissues, most of the genome is rhythmically expressed, albeit with different phase relationships within the circadian day. The individual composition of transcription factor complexes can be very specific for a given gene. Because many components of the circadian network do not cycle in phase, their relative abundance within a complex will be almost unique at any given circadian time. Depending on the stochiometry of a transcription factor complex that modulates the expression of a specific gene, the concentration of a circadian output protein would be exquisitely timed.

Different Worlds Meet at Breakfast

One of the pioneers of circadian research, Colin Pittendrigh (1919-1996), was initially sceptical about the possibility of finding clock genes in mutant screens, because he thought that circadian rhythmicity would be based on the products of too many genes. In the last decade of his scientific career, Pittendrigh recognised this mistake and became fascinated by the possibilities of applying molecular biology and genetics to understanding circadian biology. Now, it appears that Pittendrigh was correct in his notion that many genes contribute to the circadian ‘phenotype’. The molecular circadian network, as it is presently unfolding, holds many possibilities.

Viewing the clock genes and their products as individual, networked and coupled feedback loops may provide new insights into an enormously plastic system, enabling us to understand many different aspects of the circadian programme, such as individual reactions to medical interventions, jet lag and shift work. It will inform on how the circadian program influences functions of the body as different as sleep [100], digestion [101] and susceptibility to developing cancer

[14]. But the complexity and plasticity of the circadian system also means that particular care needs to be taken in interpreting results. The fact, for example, that the rat SCN appears, from data obtained using a luciferase reporter linked to *Per1* promoter sequences, to adapt to a new light cycle within a single day, while circadian behaviour (controlled by the SCN) adjusts over several days [7], may simply reflect specific aspects of *Per1* regulation — the regulation of other network components, such as *Cry* [10], may be different. Because the network's plasticity also includes the possibility of differences in different tissues, direct inferences from the circadian molecular mechanisms — for example from liver to brain — may be problematic.

The search for circadian components cannot be put to rest. There is, for example, evidence for numerous additional clock gene loci from quantitative genetic analyses [102]. So far, the success of circadian mutant screens has primarily been based on experiments in constant darkness or in rectangular light:dark cycles, while, more realistic dawn-like and dusk-like transitions have different effects on entrainment [103,104]. All factors contributing to entrainment will be instrumental in the discovery of new clock genes. Yet, recreating the 'real world' in the laboratory presents logistical and practical difficulties. Thus, searching for clock genes in the human population may be a substantial source of discovery in the coming years. The reason that family members behave so differently at breakfast (or at midnight) is determined by many factors, including age, light exposure and many genes that lie both along the circadian pathway (Figure 2D) and within the network of clock gene feedbacks (Figure 5).

Acknowledgements

We are grateful to Therese Wilson, J. Woodland Hastings, Charlambos P. Kyriacou, Urs Albrecht, Ueli Schibler and Serge Daan, for helpful comments on the manuscript. Our work is supported by the Eppendorf Company, Hamburg, by the Deutsche Forschungsgemeinschaft, the Dr.-Meyer-Struckmann-Stiftung, and by *BrainTime* from the European Commission of the European Union.

References

1. Welsh, D.K., Logothetis, D.E., Meister, M. and Reppert, S.M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron *14*, 697–706.
2. Ralph, M.R., Foster, R.G., Davis, F.C. and Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. Science *247*, 975–978.
3. Plautz, J.D., Kaneko, M., Hall, J.C. and Kay, S.A. (1997). Independent photoreceptive circadian clocks throughout *Drosophila*. Science *278*, 1632–1635.
4. Brandes, C., Plautz, J.D., Stanewsky, R., Jamison, C.F., Straume, M., Wood, K.V., Kay, S.A. and Hall, J.C. (1996). Novel features of *Drosophila period* transcription revealed by real-time luciferase reporting. Neuron *16*, 687–692.
5. Hege, D.M., Stanewsky, R., Hall, J.C. and Giebultowicz, J.M. (1997). Rhythmic expression of a PER–reporter in the Malpighian tubules of decapitated *Drosophila*: evidence for a brain-independent circadian clock. J. Biol. Rhythms *12*, 300–308.
6. Balsalobre, A., Damiola, F. and Schibler, U. (1998). A serum shock induces gene expression in mammalian tissue culture cells. Cell *93*, 929–937.
7. Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R.-I., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M. and Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. Science *288*, 682–685.
8. Yamazaki, S., Kerbeshian, M.C., Hocker, C.G., Block, G.D. and Menaker, M. (1998). Rhythmic properties of the hamster suprachiasmatic nucleus *in vivo*. J. Neurosci. *18*, 10709–10723.
9. Pittendrigh, C.S. and Daan, S. (1976). A functional analysis of circadian pacemakers in noctural rodents: IV. Entrainment: Pacemaker as clock. J. Comp. Physiol. A *106*, 291–331.
10. Reddy, A.B., Fields, M.D., Maywood, E.S. and Hastings, M.H. (2002). Differential resynchronisation of circadian clock gene expression within the suprachiasmatic nuclei of mice subjected to experimental jet lag. J. Neurosci. *22*, 7326–7330.
11. Storch, K.F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F.C., Wong, W.H. and Weitz, C.J. (2002). Extensive and divergent circadian gene expression in liver and heart. Nature *417*, 78–83.
12. Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smoth, A.G., Gant, T.W., Hastings, M.H. and Kyriacou, C.P. (2001). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr. Biol. *12*, 540–550.
13. Panda, S., Antoch, M.P., Millar, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S. and Hogenesch, J.B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell *109*, 307–320.
14. Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C.C. (2002). The circadian gene *Period2* plays an important role in tumor suppression and DNA damage responses in *vivo*. Cell *111*, 41–50.
15. Holst, E.v. (1939). Die relative Koordination als Phänomen und als Methode zentralnervöser Funktionsanalyse. Ergebn. Physiol. *42*, 228–306.
16. Swade, R.H. and Pittendrigh, C.S. (1967). Circadian locomotor rhythms of rodents in the arctic. Am. Nat. *101*, 431–466.
17. Klerman, E.B. (2001). Non–photic effects on the circadian system: results from experiments in blind and sighted individuals. In Zeitgebers, Entrainment and Masking of the Circadian System, K. Honma and S. Honma, eds. (Sapporo: Hokkaido University Press), pp. 155–169.
18. Roenneberg, T. and Foster, R.G. (1997). Twilight times - light and the circadian system. Photochem. Photobiol. *66*, 549–561.
19. Wright, K.P. and Czeisler, C.A. (2002). Absence of circadian phase resetting in response to bright light behind the knees. Science *297*, 571.
20. Freedman, M.S., Lucas, R.J., Soni, B., von Schantz, M., Munoz, M., David-Gray, Z.K. and Foster, R.G. (1999). Non-rod, non-cone ocular photoreceptors regulate the mammalian circadian behaviour. Science *284*, 502–504.
21. Lucas, R.J., Douglas, R.H. and Foster, R.G. (2001). Characterization of an ocular photopigment capable of driving pupillary constriction in mice. Nat. Neurosci. *4*, 621–626.
22. Hankins, M.W. and Lucas, R.J. (2002). A novel photopigment in the human retina regulates the activity of primary visual pathways according to long–term light exposure. Curr. Biol. *12*, 191–198.
23. Lucas, R.J., Freedman, M.S., Munoz, M., Garcia-Fernandez, J. and Foster, R.G. (1999). Non-rod, non-cone ocular photoreceptors regulate the mammalian pineal. Science *284*, 505–507.
24. Hannibal, J., Hindersson, P., Knudsen, S.M., Georg, B. and Fahrenkrug, J. (2002). The photopigment melanopsin is exclusively present in pituitary adenylate cyclase–activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. J. Neurosci. *22*, RC191.
25. Hattar, S., Liao, H.W., Takao, M., Berson, D.M. and Yau, K.W. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections and intrinsic photosensitivity. Science *295*, 1065–1070.
26. Provencio, I., Rodriguez, I.R., Jiang, G., Hayes, W.P., Moreira, E.F. and Rollag, M.D. (2000). A novel human opsin in the inner retina. J. Neurosci. *20*, 600–605.
27. Ruby, N.F., Brennan, T.J., Xie, X., Cao, V., Franken, P., Heller, H.C. and O 'Hara, B.F. (2002). Role of melanopsin in circadian responses to light. Science *298*, 2211–2213.
28. Panda, S., T.K.Sato, Castrucc, A.M., Rollag, M.D., DeGrip, W.J., Hogenesch, J.B., Provencio, I. and Kay, S.A. (2002). Melanopsin (Opn4) requirement for normal light–induced circadian phase shifting. Science *298*, 2213–2216.
29. Aschoff, J. (1959). Zeitliche Strukturen biologischer Vorgänge. Nova Acta Leopoldina *21*, 147–177.
30. Duffy, J.F., Rimmer, D.W. and Czeisler, C.A. (2001). Association of intrinsic circadian period with morningness–eveningness, usual wake time and circadian phase. Behav. Neurosci. *115*, 895–899.

31. Roenneberg, T., Wirz-Justice, A. and Merrow, M. (2003). Life between clocks - daily temporal patterns of human chronotypes. J. Biol. Rhythms *18*, 80–90.

32. Reid, K.J., Chang, A.M., Dubocovich, M.L., Turek, F.W., Takahashi, J. and Zee, P.C. (2001). Familial advanced sleep phase syndrome. Arch. Neurol. *58*, 1089–1094.

33. Ebisawa, T., Uchiyama, M., Kajimura, N., Mishima, K., Kamei, Y., Katoh, M., Watanabe, T., Sekimoto, M., Shibui, K., Kim, K. *et al*. (2001). Association of structural polymorphisms in the human *period3* gene with delayed sleep phase syndrome. EMBO Rep. *2*, 342–346.

34. Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J. and Fu, Y.H. (2001). An *hPer2* phosphorylation site mutation in familial advanced sleep phase syndrome. Science *291*, 1040–1043.

35. Carskadon, M.A., Labyak, S.E., Acebo, C. and Seifer, R. (1999). Intrinsic circadian period of adolescent humans measured in conditions of forced desynchrony. Neurosci. Lett. *260*, 129–132.

36. Carskadon, M., Wolfson, A.R., Acebo, C., Tzischinsky, O. and Seifer, R. (1998). Adolescent sleep patterns, circadian timing and sleepiness at a transition to early school days. Sleep *21*, 871–881.

37. Damiola, F., Minh, N.L., Preitner, N., Kornmann, B., Fleury–Olela, F. and Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes and Dev. *14*, 2950–2961.

38. Brown, S.A. and Schibler, U. (2001). Circadian rhythms: mop up the clock. Curr. Biol. *11*, R268–270.

39. Sweeney, B.M. and Hastings, J.W. (1957). Characteristics of the diurnal rhythm of luminescence in *Gonyaulax polyedra*. J. Cell Comp. Physiol. *49*, 115–128.

40. Michel, S., Geusz, M.E., Zaritsky, J.J. and Block, G.D. (1993). Circadian rhythm in membrane conductance expressed in isolated neurons. Science *259*, 239–241.

41. Konopka, R. and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. *68*, 2112–2116.

42. Hardin, P.E., Hall, J.C. and Rosbash, M. (1990). Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature *343*, 536–540.

43. Ralph, M.R. and Menaker, M. (1988). A mutation of the circadian system in golden hamsters. Science *241*, 1225–1227.

44. Antoch, M.P., Song, E.-J., Chang, A.–M., Hotz Vitaterna, M., Zhao, Y., Wisbacher, L.D., Sangoram, A.M., King, D.P., Pinto, L.H. and Takahashi, J.S. (1997). Functional identification of the mouse circadian *clock* gene by transgenic BAC rescue. Cell *89*, 655–667.

45. King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D.L., Hotz Vitaterna, M., Kornhauser, J.M., Lowrey, P.L. *et al*. (1997). Positional cloning of the mouse circadian *clock* gene. Cell *89*, 641–653.

46. Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S. and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

47. Albrecht, U., Sun, Z.S., Lee, C.C., Eichele, G. and McLean, V.M. (1997). A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. Cell *91*, 1055–1064.

48. Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. and Sakaki, Y. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. Nature *389*, 512–516.

49. Reppert, S.M. and Weaver, D.R. (2001). Molecular analysis of mammalian circadian rhythms. Annu. Rev. Physiol. *63*, 647–676.

50. Kume, K., Zylka, M.J., Sriram, S., Shearman, L.P., Weaver, D.R., Jin, X., Maywood, E.S., Hastings, M.H. and Reppert, S.M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell *98*, 193–205.

51. Vitaterna, M.H., Selby, C.P., Todo, T., Niwa, H., Thompson, C., Fruechte, E.M., Hitomi, K., Thresher, R.J., Ishikawa, T., Miyazaki, J. *et al*. (1999). Differential regulation of mammalian Period genes and circadian rhythmicity by *cryptochromes 1* and *2*. Proc. Natl. Acad. Sci. U.S.A. *96*, 12114–12119.

52. Darlington, T.K., Wager–Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S. and Kay, S.A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science *280*, 1599–1603.

53. Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., DeVries, G.J. and Reppert, S.M. (1998). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell *96*, 57–68.

54. Okamura, H., Miyake, S., Sumi, Y., Yamaguchi, S., Yasui, A., Muijtjens, M., Hoeijmakers, J.H.J. and van der Horst, G.T.J. (1999). Photic induction of *mPer1* and *mPer2* in *Cry*–defcient mice lacking a biological clock. Science *286*, 2531–2534.

55. Hogenesch, J.B., Gu, Y.Z., Jain, S. and Bradfield, C.A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and Hypoxia factors. Proc. Natl. Acad. Sci. U.S.A. *95*, 5474–5479.

56. Etchegaray, J.–P., Lee, C., Wade, P.A. and Reppert, S.M. (2002). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature *421*, 177–182.

57. Vielhaber, E.L., Duricka, D., Ullman, K.S. and Virshup, D.M. (2001). Nuclear export of mammalian PERIOD proteins. J. Biol. Chem. *276*, 45921–45927.

58. Shearman, L.P., Sriram, S., Weaver, D.R., Maywood, E.S., Chaves, I., Zeng, B., Kume, K., Lee, C.C., van der Horst, G.T.J., Hastings, M.H. and Reppert, S.M. (2000). Interacting molecular loops in the mammalian circadian clock. Science *288*, 1013–1019.

59. Reppert, S.M. and Weaver, D.R. (2002). Coordination of circadian timing in mammals. Nature *418*, 935–941.

60. Young, M.W. and Kay, S.A. (2001). Time zones: a comparative genetics of circadian clocks. Nat. Rev. Genet. *2*, 702–715.

61. Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U. and Schibler, U. (2002). The orphan nuclear receptor REV–ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell *110*, 251–260.

62. Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y. and Honma, K.-I. (2002). *Dec1* and *Dec2* are regulators of the mammalian clock. Nature *419*, 841–844.

63. Alvarez, J.D. and Sehgal, A. (2002). Finer clock control. Nature *419*, 798–799.

64. Gau, D., Lemberger, T., Gall, C.v., Kretz, O., Le Minh, N., Gass, P., Schmid, W., Schibler, U., Korf, H.-W. and Schütz, G. (2002). Phosphorylation of CREB Ser142 regulates light–induced phase shifts of the circadian clock. Neuron *34*, 245–253.

65. Steinlechner, S., Jacobmeier, B., Scherbarth, F., Dernbach, H., Kruse, F. and Albrecht, U. (2002). Robust circadian rhythmicity of *Per1* and *Per2* mutant mice in constant light and dynamics of *Per1* and *Per2* gene expression under long and short photoperiods. J. Biol. Rhythms *17*, 202–209.

66. Oster, H., Yasui, A., van der Horst, G.T.J. and Albrecht, U. (2002). Disruption of *mCry2* restores circadian rhythmicity in *mPer2* mutant mice. Genes Dev. *16*, 2633–2638.

67. Merrow, M., Brunner, M. and Roenneberg, T. (1999). Assignment of circadian function for the *Neurospora* clock gene *frequency*. Nature *399*, 584–586.

68. Dragovic, Z., Tan, Y., Görl, M., Roenneberg, T. and Merrow, M. (2002). Light reception and circadian behavior in 'blind' and 'clockless' mutants of *Neurospora crassa*. EMBO J. *21*, 3643–3651.

69. Aronson, B.D., Johnson, K.A. and Dunlap, J.C. (1994). The circadian clock locus *frequency*: a single ORF defines period length and temperature compensation. Proc. Natl. Acad. Sci. U.S.A. *91*, 7683–7687.

70. Froehlich, A.C., Liu, Y., Loros, J.J. and Dunlap, J.C. (2002). White Collar-1, a circadian blue light photoreceptor, binding to the *frequency* promoter. Science *297*, 815–819.

71. He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K.H. and Liu, Y. (2002). White Collar-1, a DNA binding transcription factor and light sensor. Science *297*, 840–843.

72. Crosthwaite, S.K., Dunlap, J.C. and Loros, J.J. (1997). *Neurospora wc-1* and *wc-2*: Transcription, photoresponses and the origin of circadian rhythmicity. Science *276*, 763–769.

73. Roenneberg, T. and Merrow, M. (2000). Circadian light input: omnes viae Romam ducunt. Curr. Biol. *10*, R742–R745.

74. Somers, D.E., Devlin, P.F. and Kay, S.A. (1998). Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. Science *282*, 1488–1490.

75. Roenneberg, T. and Merrow, M. (1998). Molecular circadian oscillators - an alternative hypothesis. J. Biol. Rhythms *13*, 167–179.

76. Roenneberg, T. and Merrow, M. (1999). Circadian clocks and metabolism. J. Biol. Rhythms *14*, 449–459.

77. Bognar, L.K., Adam, A.H., Thain, S.C., Nagy, F. and Millar, A.J. (1999). The circadian clock controls the expression pattern of the circadian input photoreceptor, *phytochrome B*. Proc. Natl. Acad. Sci. U.S.A. *96*, 14652–14657.

78. Roenneberg, T. and Taylor, W. (1994). Light induced phase responses in *Gonyaulax* are drastically altered by creatine. J. Biol. Rhythms *9*, 1–12.

79. Emery, P., So, W.V., Kaneko, M., Hall, J.C. and Rosbash, M. (1998). CRY, a *Drosophila* clock and light–regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell *95*, 669–679.

80. Denault, D.L., Loros, J.J. and Dunlap, J.C. (2001). WC–2 mediated WC–1–FRQ interaction within the PAS protein–linked circadian feedback loop of *Neurospora*. EMBO J. *20*, 109–117.

81. Roenneberg, T. and Merrow, M. (2002). Life before the clock - modeling circadian evolution. J. Biol. Rhythms *17*, 495–505.
82. Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K. and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science *298*, 840–843.
83. Elowitz, M.B. and Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. Nature *403*, 335–338.
84. Hoffmann, A., Levchenko, A., Scott, M.L. and Baltimore, D. (2002). The IκB–NF–κB signaling module: temporal control and selective gene activation. Science *298*, 1241–1245.
85. Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros , J., Dunlap, J., C. and Okamura, H. (1997). Light–induced resetting of a mammalian clock is associated with rapid induction of the *mPer1* transcript. Cell *91*, 1043–1053.
86. Zylka, M.J., Shearman, L.P., Weaver, D.R. and Reppert, S.M. (1998). Three *period* homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron *20*, 1103–1110.
87. Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. and Lee, C.C. (1997). RIGUI, a putative mammalian ortholog of the *Drosophila period* gene. Cell *90*, 1003–1011.
88. Lavery, J. and Schibler, U. (1998). Circadian transcription of the cholesterol 7 a hydroxylase gene may involve the liver–enriched bZIP protein DBP. Genes & Dev. *7*, 1871–1884.
89. Ripperger, J.A., Shearman, L.P., Reppert, S.M. and Schibler, U. (2000). CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. Genes Dev. *14*, 679–689.
90. Lopez–Molina, L., Conquet, F., Dubois–Dauphin, M. and Schibler, U. (1997). The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucelus and influences circadian behavior. EMBO J. *16*, 6762–6771.
91. Yamaguchi, S., Mitsui, S., Yan, L., Yagita, K., Miyake, S. and Okamura, H. (2000). Role of DBP in the circadian oscillatory mechanism. Mol. Cell. Biol. *20*, 4773–4781.
92. Krishnan, B., Levine, J.D., Lynch, M.K.S., Dowse, H.B., Funes, P., Hall, J.C., Hardin, P.E. and Dryer, S.E. (2001). A new role for *cryptochrome* in a *Drosophila* circadian oscillator. Nature *411*, 313–317.
93. Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wagner–Smith, K., Kay, S.A., Rosbash, M. and Hall, J.C. (1998). The *cry^b* mutation identifies *cryptochrome* as a circadian photoreceptor in *Drosophila*. Cell *95*, 681–692.
94. van der Horst, G.T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., AP, A.P.E., van Leenen, D. *et al.* (1999). Mammalian *Cry1* and *Cry2* are essential for maintenance of circadian rhythms. Nature *398*, 627–630.
95. Roenneberg, T. and Morse, D. (1993). Two circadian oscillators in one cell. Nature *362*, 362–364.
96. Rutter, J., Reick, M., Wu, L.C. and McKnight, S.L. (2001). Regulation of Clock and NPAS2 DNA binding by the Redox State of NAD cofactors. Science *293*, 510–514.
97. Roenneberg, T. and Merrow, M. (2002). Light reception: discovering the clock–eye in mammals. Curr. Biol. *12*, R163–R165.
98. Daan, S., Albrecht, U., van der Horst, G.T.J., Illnerova, H., Roenneberg, T., Schwartz, W.J. and Wehr, T.A. (2001). Assembling a clock for all seasons: are M and E oscillators in the genes? J. Biol. Rhythms *16*, 105–116.
99. Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., de, V., G.J. and Reppert, S.M. (1999). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell *96*, 57–68.
100. Dijk, D.-J. and Lockley, S.W. (2002). Integration of human sleep–wake regulation and circadian rhythmicity. J. Appl. Physiol. *92*, 852–862.
101. Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y. and Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. Science *291*, 490–493.
102. Shimomura, K., Low-Zeddies, S.S., King, D.P., Steeves, T.D., Whiteley, A., Kushla, J., Zemenides, P.D., Lin, A., Vitaterna, M.H., Churchill, G.A. and Takahashi, J.S. (2001). Genome–wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. Genome Res. *11*, 959–980.
103. Boulos, Z., Macchi, M. and Terman, M. (1996). Twilight transitions promote circadian entrainment to lengthening light–dark cycles. Am. J. Physiol. *271*, 813–818.
104. Wehr, T.A., Aeschbach, D. and Duncan, W.C. (2001). Evidence for a biological dawn and dusk in the human circadian timing system. J. Physiol. *535*, 937–951.
105. Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M. and Takahashi, J.S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science *288*, 483–491.
106. Bae, K., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M. and Weaver, D.R. (2001). Differential functions of *mPer1*, *mPer2* and *mPer3* in the SCN circadian clock. Neuron *30*, 525–536.